SOME STUDIES OF TYROSINASE ACTIVITY IN

STREPTOMYCES ANTIBIOTICUS

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STREPTOMYCES ANTIBIOTICUS

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CHAPTER I

INTRODUCTION

The search for particular intermediate metabolic pathways has in general followed a well-defined pattern. When alterations in the gene of an organism occur, blocking of certain metabolic pathways usually follows. Close examination of accumulated intermediates frequently reveals information regarding both the natural sequence of a metabolic pathway and the site of the genetic blocking. As early as 1918 Garrod (4) had compiled a book, *Inborn Errors of Metabolism*, in which he described single gene defects in humans that resulted in alkaptonuria, phenylketonuria, tyrosinosis, and albinism. Collecting the data available at that time he had demonstrated that homogentistic acid, that was excreted in the urine of alkaptonurics, is a normal intermediate in the oxidation of phenylalanine and tyrosine, and that alkaptonuria is due to an inability to oxidize homogentistic acid. The fundamental concept indicated by these investigations in tyrosine and phenylalanine metabolism is that a single gene mutation can only block a single metabolic reaction.
A mutant strain of a microorganism may be compared with a human being having a genetic defect in that both have lost the ability to perform a particular metabolic reaction which normally produces a product essential for growth or for some other phenotypic expression. Unlike human mutants, mutant strains of many microorganisms can readily be produced by ultraviolet radiation, X-ray radiation, and chemical mutagens.

It has been reported by Waksman (12) that mutations of the actinomycetes group of microorganisms probably depend on additions, deletions or rearrangements of chromosomal material exclusive of mere recombinations. At the ultimate molecular level the alteration (mutation) may involve a substitution of one nucleotide for another; thus, the formation of new strains through mutations is fundamental and hereditary. Mutations of actinomycetes as reported by Waksman also lead to complete changes of one or more of the characters such as the appearance or disappearance of certain physiological or morphological properties, notably nature of the pigments, formation of specific enzymes, capacity to cause infection, production of aerial mycelium, and manner of sporulation.

The soil actinomycete, *Streptomyces antibioticus*, was chosen as an object to study the biosynthetic pathway of the
antibiotic, actinomycin, since it produces various forms of actinomycin on a chemically defined medium. According to the nomenclature of Waksman, *Streptomyces antibioticus* is pigmented deep brown to black on proteinaceous media, is melanin positive, and grows a cream to brown colored colony that produces sporophores in cluster. On a nutrient agar it produces gray to yellowish green aerial mycelium. Since the first isolation and crystallization of actinomycin by Waksman and Woodruff(13) in 1940 from *Streptomyces antibioticus*, the actinomycins have gained much attention among research workers due to two important reasons. First started in 1952, the work of Hackmann (5), as well as the observations of others, has indicated that actinomycin exerts an effect on experimental tumors and may be used as an antibiotic or chemotherapeutic agent. Second, the two forms of actinomycin, C and D, (see Figure I) have been highly useful as chemical tools in the study of protein biosynthetic mechanisms. The compound is reported to inhibit the biosynthesis of the genetic material, ribonucleic acid (RNA), by binding with deoxyribonucleic acid (DNA), rendering the DNA-directed RNA synthetic process inactive. Because of this inhibition, actinomycin has been known to suppress the growth of viruses constituted of DNA but not RNA.
The isolation and structure of several forms of actinomycin from \textit{S. antibioticus} have been reported by Johnson.

![Chemical Structure of a Typical Actinomycin (Actinomycin D)](image)

\textbf{Fig. 1--Chemical Structure of a Typical Actinomycin (Actinomycin D)}

(6). Actinomycin is an antibiotic with a phenoxazinone ring structure to which are attached two pentapeptides. The various types of actinomycins depend on the amino acid sequence of the two pentapeptides. The nature of the condensation reaction
that couples the pentapeptides to the chromophoric ring struc-
ture and the sequence of reactions involved in the pentapep-
tide synthesis have not been elucidated to this date. It is known that tryptophan (3) and 4-methyl-3-hydroxyanthranilic acid (11) serve as precursors to the phenoxyazinone ring struc-
ture, but the stepwise sequence of reactions leading to the formation of the ring structure has not been determined.

Since the successful demonstration of Beadle and Tatum in 1941 (2) in which mutant strains of *Neurospora crassa* were employed to elucidate biochemical pathways in carbohydrate, lipid, and protein metabolism, it was the initial proposal in this study to induce and isolate a specific actinomycinless mutant of *S. antibioticus* in which the intermediate reactions involved in the biosynthesis of actinomycin could be studied.

N-Methyl-N'-nitro-N-nitrosoguanidine was chosen as a chemomutagenic agent because this compound produces multiple changes in the genetic information at extremely low kill rates. Since there is nothing to suggest what parts of the genetic information should be altered to block actinomycin production, it is instinctively felt that the best mutagen should be one which could affect multiple sites; previous successes with the above mutagen have been reported (7-9). The object here is to induce a mutant incapable of synthesizing actinomycin.
A series of possible intermediates can then be added to the growth medium to see which one will be capable of restoring the end product production. By use of different actinomycinless mutants and the determination of specific compounds exhibiting a positive response in actinomycin production, a probable biosynthetic pathway might be proposed for this antibiotic.

In the studies to be described, N-methyl-N'-nitro-N-nitrosoguanidine produced mutant strains that exhibit an apparent increase in actinomycin production, but in numerous experiments this mutagenic agent failed to produce a mutant strain incapable of actinomycin production on a defined medium. However, several mutants were isolated which did not produce the black melanin pigment characteristic of the non-mutant wild type in either Trypticase Soy (TS) or Emerson's Broth (EM) media. This finding suggested the blocking of another entirely different pathway, namely, the formation of melanin from tyrosine via 3,4-dihydroxyphenylalanine (DOPA). The enzyme, tyrosinase, is known to be intimately involved in melanin formation- -quite possibly the mutations producing melaninless mutants were the result of alterations in the gene directing the biosynthesis of tyrosinase.

Tyrosinase activity from a wide variety of sources has been known and studied for many years. An excellent review
by Nelson and Dawson (10) revealed the wide occurrence of tyrosinase in plant and animal sources. It has been studied in potato, dahlia bulb, Indian bean, wheat bran, the common mushroom (*Psalliota campestris*) and the wild mushroom (*Lactarius piperatus*). It has been isolated from animal sources such as the skins of rabbits, of various colored races, the ink sac of the cuttlefish, the blood of arthropods, and the blood of the octopus. Tyrosinase is also present in bacteria (*B. niger*), insects, and mutants of the fungus, *Glomerella*. It will act upon a variety of substrates such as adrenaline, phenol, catechol, m- and p-cresol. However, the best known reaction of tyrosinase in microorganisms and higher plants and animals is the production of melanins from tyrosine or from DOPA. Up to 1952, DOPA oxidase was considered the enzyme which catalyzes the formation of melanins from DOPA. Lerner (8) established in a series of studies that tyrosinase is responsible for the entire process.

Tyrosinase is a copper protein, and in the oxidation of tyrosine to DOPA and DOPA quinone copper goes through the cycle, \( \text{Cu}^+ + \text{O}_2 \rightarrow \text{Cu}^{++} \), \( \text{Cu}^{++} + \text{e}^{-} \rightarrow \text{Cu}^+ \), during which it acts as a catalyst of the oxidation. The manometric method of measuring the uptake of oxygen was employed in the early studies of tyrosinase. The agar gel electrophoresis method
of assay was occasionally used, but the studies now are done mostly with the spectrophotometric method. When melanin is formed in the presence of protein, the polymerized pigment is firmly attached to protein in the form of an insoluble granule and can be detected spectrophotometrically by changes of spectrum accompanying the oxidation of tyrosine. A proposed reaction sequence from tyrosine to melanin is outlined on the following page in Figure 2.

The study reported herein concerns itself with the isolation of melaninless mutants of *S. antibioticus*, ATCC, 3723, and with the demonstration that at least one of the sites of mutation involves that of the enzyme, tyrosinase. The tyrosinase activity of non-mutant *S. antibioticus* was concentrated up to 200 fold by a novel enzyme purification procedure. The specificity of the purified enzyme for several potential substrates was also determined and the results are described.
Fig. 2—Conversion of Tyrosine to Melanin catalyzed by Tyrosinase.
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CHAPTER II

EXPERIMENTAL

Growth and Culture of *Streptomyces antibioticus*

A culture of *Streptomyces antibioticus*, ATCC, 3723, was obtained from the American Type Culture Collection. A culture of *Streptomyces antibioticus*, I.S.P. 5324 was obtained from a stock culture from the Department of Biology, North Texas State University. The cultures were maintained on agar slants of a defined medium (DF) as described by Katz (1), or on agar slants of either of two proteinaceous-enriched media, Emerson's Broth (EM) and Trypticase-Soy Broth (TS). Periodic transfers to fresh stock media were necessary to maintain viability of the organisms.

In the liquid DF medium *S. antibioticus* will generally produce actinomycins after 48 to 72 hours. The actual incubation time required for formation of the antibiotic varies considerably, however, and on occasions as little as 24 hours or as many as 100 hours are required. The size, shape and color of individual colonies in the defined medium are generally small, white and spherical. On solid DF medium yellowish colonies result after approximately two days incubation.
In either TS or EM liquid medium a dark melanin-like pigment is produced by the organism after one to two days incubation, and the medium continues to darken with longer incubation times. Growth of the organism is very rapid in either of these proteinaceous media. When large masses of *S. antibioticus* were required for preparation of cell-free extracts, either EM or TS media were used.

**Mutant Technique**

A fresh culture of *S. antibioticus*, ATCC, 3723, on a TS agar slant was placed in an incubator at 37 degrees and incubation was continued for three to four days, or until secondary growth mycelium appeared. Ten ml of sterile 0.9 percent saline was then added to the slant tube. Mycelia were detached from the colonies by use of a sterilized wire loop or by gentle shaking. The saline solution containing the detached mycelia was then poured into a flask containing 100 ml of sterile saline and placed in a water bath at 37 degrees for a few minutes. A 10 ml aliquot was then removed and placed in an empty sterile test tube (to serve as a control for the determination of percent kill). Then 0.5 mg/ml of the chemomutagenic agent, N-methyl-N'-nitro-N-nitrosoguanidine was added to the flask. Both the mutation flask and the control tube were incubated in the water bath at 37 degrees for 1.5
hours. Then serial dilutions of both the mutation medium and the control medium (one ml of dilutions ranging from $10^{-1}$ to $10^{-4}$) were added to petri dishes containing either sterile TS agar or sterile DF agar. The petri dishes were then placed in an incubator at 37 degrees, and incubation was continued until distinct colonies appeared. Lethal mutations caused by the chemomutagen were generally on the order of 50 to 70 percent of the cell population.

Isolation of the Mutants

Mutants were selected on the basis of color production. Non-mutant colonies growing on DF medium agar become yellow due to the production of actinomycin; therefore, colonies remaining colorless (or white) after this time interval were sought as possible actinomycinless mutants. Although several such colorless colonies were found during the course of the mutation studies, removal and subculturing invariably led to an actinomycin-positive colony. This indicates a probable spontaneous reversion to the wild type. More success was obtained in the isolation of melaninless mutants of S. antibioticus. Albino (white) colonies growing on TS agar plates (derived from cells exposed to the chemomutagen) were removed and subcultured in either TS or EM medium. After several subcultures, those colonies which remained incapable
of producing melanin pigment were given a mutant number, streaked on stock agar slants, incubated at 37 degrees until a sufficient growth had occurred, and then stored at 5 degrees for future study.

Growth of *S. antibioticus* for Enzyme Isolation

A single colony with mycelia (from an EM agar slant) was inoculated into 100 ml of sterile liquid EM broth, and the inoculated medium was then placed on a shaker at room temperature for two to three days to allow for luxurient growth. At this time the entire cell mass was used to inoculate 500 to 1000 ml of sterile EM medium under asceptic conditions, and incubation with shaking was continued for two to three more days. The cell culture was removed from the shaker and allowed to stand at room temperature for two to five days without any agitation. During the overall incubation the color of the growth medium changed progressively from yellow to brown to almost black. Highest levels of tyrosinase were obtained from cells grown under these conditions.

Assays of Tyrosinase Activity

The assay of tyrosinase activity involves the spectrophotometric measurement of pigment formation (melanin) from DL-3, 4-dihydroxyphenylalanine (DOPA). In general, the assay
reaction mixture contained per 3.0 ml: DL-DOPA, 50 μmoles; tris-(hydroxymethyl) amino methane (Tris) buffer, pH 7.5, 100 μmoles; and enzyme (at various stages of purification and in rate limiting amounts). The increase in absorbance was followed at 400 μm employing a Beckman DBG recording spectrophotometer. The velocity of the enzymatic reaction was expressed in terms of change in optical density per minute. Specific activity was calculated on the basis of change in optical density per minute per mg of protein. Protein concentrations were determined on all enzyme fractions by the spectrophotometric method of Warburg and Christian (2).

Isolation and Purification of Tyrosinase

Both of the non-mutant (herein considered wild type) strains of *Streptomyces antibioticus* (strain ATCC 3723 and I.S.P. 5324) produce high levels of tyrosinase when grown on proteinaceous media. Purification of tyrosinase activity was attempted on both strains, and similar fractionation steps with either strain gave similar purifications. The procedure of enzyme purification given below is therefore generally applicable to both of the mutant strains.

Cells grown as described above were harvested from EM broth by filtration on a paper filter. After washing with distilled water the cell mass was compacted by centrifugation
at 10,000 x g for five minutes in a high speed refrigerated centrifuge. The compact cell mass was then weighed, and 2.5 volumes of 0.02 M Tris buffer, pH 7.5, were added. The cells were then disrupted by sonication (Branson Sonifier) for five minutes at 0 degrees. Unbroken cells and cell debris were removed by centrifugation at 20,000 x g for 25 minutes at 0 degrees. The clear supernatant, termed "crude enzyme", was utilized for further purification.

To one volume of crude enzyme at 0 degrees was added (slowly with stirring) one-tenth volume of 10 percent streptomycin sulfate. The solution was stirred in the cold for an additional 20 minutes to insure complete precipitation of the streptomycin sulfate-nucleic acid-protein complex. The precipitate, which contained most of the tyrosinase activity, was removed by centrifugation, and then dispersed in one volume (equal to the original volume of the crude enzyme) of 0.02 M Tris buffer, pH 7.5. The resulting supernatant solution contained virtually all the tyrosinase activity and had a specific activity approximately twenty-fold greater than the original crude enzyme.

Further purification was accomplished by use of anion exchange chromatography. The anion exchange material, diethylamino ethyl Sephadex (Sephadex A-25, coarse), was prepared for
use as follows:

1. Sephadex beads were allowed to swell several hours in water and the fines were removed by decantation.

2. The beads were filtered, washed on the filter with 0.5 N hydrochloric acid, followed by washing with water.

3. The beads were then suspended in 0.5 N sodium hydroxide, filtered, and again washed with water.

4. The beads were again resuspended in water, and acid was added until the water was neutral. The beads were then filtered, washed with water, and finally resuspended in the appropriate buffer for use in the purification of tyrosinase.

The washed Sephadex A-25 beads were then packed into a column (1 cm x 25 cm) and equilibrated with 0.02 M Tris buffer, pH 7.5. A solution of the partially purified enzyme (streptomycin sulfate step) was then added to the column in the ratio of 1.0 mg of protein per cm of column height. The desired enzyme activity was eluted from the column employing Tris buffer, pH 7.5 as eluent with a gradient of 0.02 M to 0.1 M. Total elution volume was approximately 300 ml and individual fractions of 15 ml each were collected. Each fraction was then assayed for tyrosinase activity, and after protein concentrations were determined, the specific activities were calculated. Those fractions having the highest specific
activities for tyrosinase were pooled and stored at -5 degrees for future study. Overall purification from the crude enzyme was on occasion up to 200-fold.

Semi-Quantitative Determination of Actinomycin

Actinomycin was determined and its relative concentration estimated by a spectrophotometric analysis of ethyl acetate extracts.

The growth medium still containing cells of *Streptomyces antibioticus*, was extracted three times with 0.2 volumes of ethyl acetate. The extracts were combined, dried with anhydrous sodium sulfate, and reduced to a predetermined volume. The absorption spectrum between 400 m\(\mu\) and 500 m\(\mu\) was determined, employing a Beckman DBG recording spectrophotometer (ethyl acetate was employed as a blank). The characteristic absorptions for the actinomycins lie between 430 and 460 m\(\mu\).
CHAPTER BIBLIOGRAPHY


CHAPTER III

RESULTS AND DISCUSSION

Several mutational experiments were conducted using the methods described in the experimental section. No stable actinomycinless mutants could be isolated, although a few colonies were isolated which initially produced decreased levels of actinomycin. On continued subculture these isolates could not be distinguished from the parent strain with respect to actinomycin production. Several mutants were isolated which were incapable of producing melanin pigment in proteinaceous medium, and this impairment was not lost in these mutants on continued subculture. Three of the mutant strains isolated are listed below.

Mutant 80—This mutant was isolated on TS medium. It produces actinomycin on defined medium, but no melanin-like pigmentation occurs on any of the enriched media employed. The growth pattern is basically the same as *Streptomyces antibioticus*, ATCC, 3723, but the rate of growth of the mutant is often more rapid.

Mutant O. Y.—This mutant was isolated on defined medium, and it represents a very active actinomycin-producing strain.
It will not pigment in enriched media. The growth pattern and rate of growth are basically the same as *Streptomyces antibioticus*, ATCC, 3723.

**Mutant C-11**—This mutant was isolated on defined medium, and it was selected originally as a comparison mutant for Mutant O.Y. in regard to actinomycin production. On the subsequent transfer that followed it proved to be an excellent actinomycin-producing strain itself. It will not pigment on enriched medium. The rate and pattern of growth appears the same as *Streptomyces antibioticus*, ATCC, 3723.

Spectrophotometric analysis of actinomycin production of *Streptomyces antibioticus*, Mutant 80, Mutant O.Y., and Mutant C-11 have been made in concentrated ethyl acetate extracts that were taken from both defined and TS growth media. Characteristic absorptions for actinomycin were recorded in all mutants in all media. Of major significance is that, even though the mutant strains isolated do not produce melanin-like pigmentation in enriched medium, they do continue to produce actinomycin in both defined and enriched media. Table I below summarizes the findings obtained and emphasizes the lack of correspondence of actinomycin production and melanin-like pigment production in mutants isolated.

Sevick observed in 1957 (1) a direct relationship between the formation of actinomycin and phenol oxidase. He
### TABLE I

**ACTINOMYCIN AND MELANIN PIGMENT PRODUCTION IN MUTANT AND NON-MUTANT STREPTOMYCES ANTIBIOTICUS**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Medium</th>
<th>Actinomycin* Production</th>
<th>Pigment** Production</th>
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<tr>
<td><strong>Streptomyces</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>antibioticus</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATCC, 3723</td>
<td>DF</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>TS</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>EM</td>
<td>N.D.***</td>
<td>+</td>
</tr>
<tr>
<td><strong>Streptomyces</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>antibioticus</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mutant 80</td>
<td>DF</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>TS</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>EM</td>
<td>N.D.</td>
<td>-</td>
</tr>
<tr>
<td><strong>Streptomyces</strong></td>
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<tr>
<td><em>antibioticus</em></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Mutant O. Y.</td>
<td>DF</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>TS</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>EM</td>
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<tr>
<td><strong>Streptomyces</strong></td>
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<tr>
<td><em>antibioticus</em></td>
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<tr>
<td>Mutant C-11</td>
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<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>TS</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>EM</td>
<td>N.D.</td>
<td>-</td>
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</tbody>
</table>

*See experimental section for the semi-quantitative determination of actinomycin.

**Pigment production was based on visual observation.

***N.D.—Actinomycin production was not determined.

Postulated on the basis of these observations that phenol oxidase of the tyrosinase type most probably was involved in
the biosynthesis of actinomycin. The observations of the study herein described suggest that tyrosinase activity associated with melanin formation is not involved in actinomycin production. However, on several occasions, due to variation in growth, the wild type Streptomyces antibioticus produced abnormally high actinomycin levels in EM medium; in these instances cell extracts apparently had a lower than usual tyrosinase activity.

It is possible that when actinomycin production is abnormally high, tyrosinase activity is relatively low, and vice versa. One explanation for this finding would be that tyrosinase activity is inducible and, when actinomycin production is high, substrates capable of inducing tyrosinase formation are either not produced themselves or are channeled into actinomycin formation. That tyrosinase is inducible is supported by the finding that young cultures of wild type S. antibioticus have a relatively low tyrosinase activity. Tyrosinase activity rises to a high level only in old cultures. Sizer (2) reported that although other enzymes such as the cytochrome system or peroxidase can produce melanin from tyrosine or DOPA, it is usually tyrosinase which is associated with melanin formation in living systems. In order to determine whether a quantitative difference exists in the tyrosinase
activity of *S. antibioticus*, wild type (ATCC, 3723) and Mutant 80 isolated therefrom, cell-free extracts of these organisms were prepared and tyrosinase activity was determined. The results of this study are shown in Table II.

**TABLE II**

RELATIVE TYROSINASE ACTIVITY* OF WILD TYPE *S. ANTIBIOTICUS* AND MUTANT 80

<table>
<thead>
<tr>
<th>Protein conc. (mg/ml)</th>
<th>ΔO.D./min</th>
<th>Specific Activity**</th>
<th>Protein conc. (mg/ml)</th>
<th>ΔO.D./min</th>
<th>Specific Activity**</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.27</td>
<td>0.010</td>
<td>0.037</td>
<td>1.2</td>
<td>0.004</td>
<td>0.0033</td>
</tr>
<tr>
<td>0.54</td>
<td>0.019</td>
<td>0.035</td>
<td>1.8</td>
<td>0.007</td>
<td>0.0038</td>
</tr>
<tr>
<td>1.08</td>
<td>0.032</td>
<td>0.030</td>
<td>2.4</td>
<td>0.0083</td>
<td>0.0035</td>
</tr>
</tbody>
</table>

*The assay procedure for tyrosinase activity is given in the experimental section.

**Specific activity is in terms of change in optical density per minute per mg of protein.

It is observed that the specific activity of tyrosinase in Mutant 80 is approximately one-tenth that of the wild type parent strain. There is obviously a correlation between the ability to produce a melanin-like pigment during growth on enriched medium and the ability to oxidize DL-DOPA to melanin in cell-free extracts.
Since further studies were anticipated on the tyrosinase of the wild type organism, optimum conditions for assay of the rate of pigment production catalyzed by this enzyme were determined. Although variations of pH from 5.5 to 8.0 had little effect on tyrosinase activity, variations in the concentration of Tris buffer, pH 7.5, produced a marked effect. Figure 3 shows the effects of various concentrations of Tris buffer on the specific activity of the enzyme. It can be seen that when the buffer concentration is very low the specific activity is highest; higher buffer concentrations tend to inhibit tyrosinase activity.

The effect of substrate concentration (DL-DOPA) on the rate of the enzymatic reaction was determined, and the results are plotted in Figure 4. It can be seen that at the level of approximately two μmoles per ml of DL-DOPA the rate of reaction is almost completely independent of substrate concentration. To insure complete saturation of the enzyme, when assays for enzyme activity were conducted, a DL-DOPA concentration of 8.3 μmoles/ml was usually employed. It was of interest to study other properties of the tyrosinase of wild type S. antibioticus, and purified preparations of the enzyme were desirable. Several methods of protein purification were tested, such as ammonium sulfate fractionation,
Fig. 3--The effect of Tris buffer concentration at pH 7.5 on the specific activity of Tyrosinase. The assay system is described in the experimental section. The concentration of partially purified enzyme was 0.16 mg per ml of reaction mixture. Specific activity is in terms of change in optical density per minute per mg of protein.
Fig. 4—The effect of DL-3, 4-Dihydroxyphenylalanine concentration on the specific activity of Tyrosinase. The assay system is described in the experimental section. The concentration of DL-DOPA ranged from 1.67 to 16.7 μmoles per ml. Specific activity is in terms of change in optical density per minute per mg of protein.
absorption on calcium phosphate gel, and DEAE-cellulose; however, purifications above 100-fold (up to 200-fold) were readily obtained by use of two relatively simple fractionation procedures—namely, streptomycin sulfate precipitation followed by anion exchange chromatography employing DEAE-Sphadex, A-25. The detailed procedures involved in the two-step process are given in the experimental section. Table III presents a summary of the increase in specific activity obtained with each purification step in a representative experiment.

### TABLE III

**PURIFICATION OF TYROSINASE ACTIVITY* FROM STREPTOMYCES ANTIBIOTICUS**

<table>
<thead>
<tr>
<th>Enzyme Fraction</th>
<th>Protein Concentration (mg/ml)</th>
<th>Yield (percent)</th>
<th>Specific Activity**</th>
<th>Fold Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Crude Enzyme</td>
<td>45.00</td>
<td>100</td>
<td>0.00196</td>
<td>0</td>
</tr>
<tr>
<td>II. Streptomycin Sulfate Supernatant</td>
<td>2.10</td>
<td>65</td>
<td>0.0306</td>
<td>16</td>
</tr>
<tr>
<td>III. DEAE-Sephadex</td>
<td>0.016</td>
<td>11</td>
<td>0.210</td>
<td>110</td>
</tr>
</tbody>
</table>

*The assay procedure for tyrosinase activity is given in the experimental section.

**Specific activity is in terms of change in optical density per minute per mg of protein.
The most highly purified fractions of tyrosinase were employed for studies of substrate specificity. Of several possible substrates tested, DL-3, 4-dihydroxyphenylamine (DOPAmine) was most active. Tyrosine and tyramine were acted upon by the enzyme only after extended incubation periods.

Kinetic data obtained from a study of purified tyrosinase activity on DL-DOPA have been employed for the determination of the Michaelis constant, $K_m$. Figure 5 shows a Lineweaver-Burk plot of kinetic data of Tyrosinase activity on DL-DOPA. The assay procedure is described in the experimental section. The concentration of DL-DOPA ranges from 1.67 to 16.7 μmoles per ml.
Burk plot of those kinetic data; an extension of the straight line plot obtained gives rise (the reciprocal of the intercept) to a Km value of $2.6 \times 10^{-4}$ M for DL-DOPA. This value would, as a first approximation indicate a fairly high affinity of DL-DOPA for the tyrosinase of _S. antibioticus_.

In conclusion, the findings of this study indicate that tyrosinase activity is responsible (at least it has a significant role) for melanin pigment production in _S. antibioticus_, ATCC 3723 and _S. antibioticus_, I.S.P. 5324; the failure of the mutant organisms to produce melanin pigment has been correlated with their extremely low tyrosinase activity.
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